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(54) Title: BACTERIOPHAGE-MEDIATED LYSIS ANTHRAX BACTERIA

(57) Abstract: The present invention provides a recombinant lytic bacteriophage, said bacteriophage being adapted to express at least one non-structural protein which is adapted to cause lysis of anthrax bacteria and at least one non-structural protein which is adapted to inhibit anthrax toxin activity ; a pharmaceutical composition including such a bacteriophage ; and a method for the treatment of anthrax in a host, comprising the step of administering to said host a recombinant bacteriophage in accordance with the invention, which bacteriophage is lytic to at least one strain or to a mixture of strains of bacteria within said host.

BACTERIOPHAGE-MEDIATED LYSIS ANTHRAX BACTERIA

The present invention relates to the treatment of anthrax infection, particularly in vivo anthrax infection.

Anthrax is an acute infective and often fatal disease affecting both humans and a variety of animals, including both wild and domestic animals. As with any serious disease, improved methods and means for therapy are prima facie desirable. In the case of anthrax, the severity and virulence of the disease renders the development of effective and mass-producible medicaments a matter of urgency.

Anthrax is characterised by systemic infection with the anthrax bacillus, *Bacillus anthracis*. Typically, systemic anthrax infection is initiated by the entry of *Bacillus anthracis* endospores into the body of a human or animal host through abrasions in the skin, through inhalation, or through ingestion of infected consumables. Following host invasion, the endospores are phagocytosed by host macrophages and are carried to regional lymph nodes. After a period of incubation within the phagocytotic vesicles of the macrophage, which may be as short as thirty minutes, the endospores germinate into vegetative anthrax bacteria which are released into the macrophage cytoplasm (Hanna PC, 2001, 4th International Congress on Anthrax, June 10-13, Annapolis, Maryland, USA).

The macrophage cytoplasm provides a viable environment for bacterial replication, and after a period of multiplication and incubation the bacteria are released from the macrophages, and enter the lymphatic system and bloodstream of the host, quickly resulting in systemic bacterial infection. The toxic effects of anthrax infection are produced through the activity of anthrax toxin, which is expressed by vegetative bacteria from the early stages of germination (Noskov et al, 2001, Board 34B, 4th International

Congress on Anthrax, June 10-13, Annapolis, Maryland, USA). Anthrax toxin constitutes three proteins, namely protective antigen (PA), edema factor (EF) and lethal factor (LF), which combine to form edema toxin (PA/EF) and lethal toxin (PA/LF).

As reported in US 5677274 and the references thereto, PA is expressed by anthrax bacteria as an 83kDa moiety (PA-83) which is capable of binding to a specific receptor on the surface of macrophages, monocytes and other susceptible host cells (Leppla et al, Abstract, Fifth European Workshop on Bacterial Protein Toxins, Veldhoven, June 30-July 5 1991). Following binding to this receptor, PA-83 is susceptible to site-specific cleavage by a protease expressed on the surface of the host cell (probably furin), which results in the removal of a 19kDa polypeptide fragment from PA-83. This reduces the antigen size to 63kDa (PA-63), and exposes a binding site on PA-63 with high affinity for LF and EF. Receptor-bound PA-63 is therefore capable of binding either LF or EF, in competition with one another.

Studies indicate that receptor-bound PA-63 acts as an intracellular delivery vehicle for delivering bound LF or EF into the interior of the host cell (Blaustein et al, *Proc. Natl. Acad. Sci. USA* 86:2209-2213, 1989; Koehler & Collier, *J. Mole. Microbiol.* 5:1501-1506, 1991). Whilst extracellular LF and EF demonstrate no toxicity towards eukaryotic cells, the PA-mediated importation of these factors into host cells such as macrophages enables powerful cytotoxic action against the host cells. Studies have shown that LF is capable of killing macrophages within two hours of PA-mediated importation, through the cleavage of intracellular kinases Mek1, Mek2 and Mek3 (Moayeri M, 2001, 4th International Congress on Anthrax, June 10-13, Annapolis, Maryland, USA).

In the early stages of anthrax disease progression, before the development of symptoms, vegetative *B. anthracis* bacteria are confined to

macrophages and lymph nodes, where they secrete lethal toxin, damaging local cells and tissues. However, as the bacterial infection spreads within the host and becomes systemic, lethal toxin accumulates in the bloodstream and spreads to all parts of the host body, resulting in massive septicemia and rapid death.

Hitherto, attempts to combat and treat anthrax have pursued two main strategies. Firstly, attempts have been made to eliminate the cause of anthrax infection, through targeting infective anthrax bacteria within an infected host using antibiotics. For example, antibiotics such as doxycycline, ciprofloxacin and penicillin are capable of treating early-stage anthrax infection when administered in large doses (up to 1g/day for 14-30 days). However, the rapid rate of anthrax expansion in vivo means that antibiotics quickly become ineffective as a means of eradicating infection, and accordingly unless anthrax infection is diagnosed at a very early stage, before the development of any symptoms, the administration of antibiotics in an attempt to cure the disease is largely futile.

Evidently, this poses severe limitations on the usefulness of antibiotics for treating anthrax. Moreover, penicillin-resistant strains of *B. anthracis* have already been identified as clinical isolates (Lalitha & Thomas, 1997, Penicillin resistance in *B. anthracis* *Lancet* 349:1522), and there remains the potential for chance or deliberate development of further antibiotic-resistant strains, hence further reducing the desirability of relying on antibiotics alone for the treatment of anthrax.

A second line of defence against anthrax infection involves the direct targeting and disablement of anthrax toxin. As the toxicity of both EF and LF is reliant on PA-mediated cytoplasmic delivery, attempts have been made to inhibit the delivery function of PA through blocking PA binding to target host cells. In one study, several human single-chain Fv antibody fragments (scFv) were isolated from a naive antibody library synthesised from non-

immunised human rearranged V-genes, biopanned against PA83, and characterised for their capacity to disrupt receptor-mediated binding of PA (Cirino et al, 1999, Disruption of Anthrax Toxin Binding with the use of Human Antibodies and Competitive Inhibitors, *Infect. and Immun*, 67(6):2957-2963). However, the efficacy of such fragments for inhibiting anthrax toxin activity in vivo, when administered directly to an infected host, has been found to be limited.

Accordingly, there is an urgent need for an improved means for treating anthrax infection, particularly in vivo anthrax infection. In particular, there is a need for a means for treating anthrax infection which will be capable of successfully combatting the disease at an advanced stage (after the development of symptoms).

According to one aspect of the present invention therefore, there is provided a recombinant lytic bacteriophage, said bacteriophage being adapted to express at least one non-structural protein which is adapted to cause lysis of anthrax bacteria and at least one non-structural protein which is adapted to inhibit anthrax toxin activity.

A bacteriophage in accordance with the invention can be readily obtained by genetic modification of the genome of an existing bacteriophage, such as a wild-type bacteriophage, such as to insert the coding sequences of said non-structural proteins. The recombinant bacteriophage can be administered to a host, where it can infect resident bacteria and insert its genome for expression within the infected bacteria. This will enable synthesis of said non-structural proteins within the infected bacteria, at a rate directly related to the rate of replication of the bacteriophage. The non-structural proteins will be released into the host for anti-anthrax action following lysis of the bacteria by the bacteriophage. The two-pronged attack thereby mediated by the recombinant bacteriophage against both anthrax bacteria and anthrax toxin enables efficient treatment of anthrax infection.

According to another aspect of the present invention, there is provided a pharmaceutical composition comprising a recombinant bacteriophage in accordance with the present invention and one or more pharmaceutically acceptable excipients or carriers.

According to yet another aspect of the present invention, there is provided a method for the treatment of anthrax in a host, comprising the step of administering to said host a recombinant bacteriophage in accordance with the present invention, which bacteriophage is lytic to at least one strain or to a mixture of strains of bacteria within said host. In particular, there is provided a method for the treatment of anthrax infection in a human or animal, comprising the step of administering to said human or animal an effective amount of a pharmaceutical composition in accordance with the invention.

According to yet another aspect of the present invention, there is provided a method for the use of a bacteriophage in accordance with the invention in the production of a medicament for use in the treatment of anthrax infection.

Advantageously, said bacteriophage may be lytic to said anthrax bacteria. Thus, the rate of expansion of said bacteriophage, and hence the rate of production of said non-structural proteins, will increase in the presence of a greater quantity of anthrax bacteria. Accordingly, following administration of said bacteriophage to a host infected with anthrax bacteria, the amount of said non-structural proteins with anti-anthrax action produced in the host will be determined by the extent of anthrax infection within the host, hence optimising the efficiency of treatment, even at an advanced stage of the disease.

In especially preferred embodiments, therefore, said bacteriophage may comprise lytic anthrax gamma-phage or bacteriophage CP-51. Lytic anthrax gamma-phage has been shown to have a high degree of specificity

towards *B. anthracis* (Abshire et al. 2001 Board 29B, Validation of the use of gamma phage for identification of *Bacillus anthracis*, 4th International Congress on Anthrax, June 10-13, Annapolis, Maryland, USA). Other non-anthracis strains, in particular *B. cereus* ATCC 4342 and *B. mycoides* CDC 680, are also susceptible to infection by gamma-phage and can be used for accumulation of the phage. Similarly, bacteriophage CP-51, which is lytic for *B. anthracis*, can be produced in quantity in *B. cereus*.

Advantageously, at least one of said non-structural proteins adapted to cause lysis of said anthrax bacteria may comprise the binding and catalytic domains of a phage lysin with specificity for said anthrax bacteria. Phage lysin is an enzyme produced by lytic phage, which is capable of binding to bacteria and initiating lysis thereof. Unlike antibiotics, phage lysin can target and lyse both dormant and growing bacterial cells, and may also be highly specific for particular bacterial strains. In especially preferred embodiments, at least one of said non-structural proteins adapted to cause lysis of said anthrax bacteria comprises a phage lysin with specificity for said anthrax bacteria.

Optionally, however, at least one of said non-structural proteins which is adapted to cause lysis of said anthrax bacteria may comprise an antibiotic, such as penicillin, ciprofloxacin or doxycycline.

Advantageously, said bacteriophage is adapted to express at least two non-structural proteins which are capable of causing lysis of anthrax bacteria. Thus, where a strain of anthrax has or develops immunity to one of the lysins expressed by the bacteriophage, lysis of the anthrax bacteria may still be effected through the action of the second or further lysins expressed by the bacteriophage.

In preferred embodiments of the invention, at least one of said non-structural proteins which is adapted to inhibit anthrax toxin activity may comprise at least one PA binding inhibitor peptide which is adapted to

inhibit a binding activity of the protective antigen moiety of said anthrax toxin. Said PA binding inhibitor peptide may for example be adapted to inhibit binding of said protective antigen moiety to eukaryotic cells such as macrophages and/or monocytes, such as to prevent the importation into said eukaryotic cells of one or more moieties of said anthrax toxin having cytotoxic activity, such as the lethal factor moiety or edema factor moiety of said anthrax toxin.

In one aspect of the invention, said PA binding inhibitor peptide may comprise a peptide with high binding affinity for a binding site on said protective antigen moiety, which peptide is arranged to bind to said binding site such as to prevent the binding of other polypeptides, such as receptors, to said binding site.

Thus, said PA binding inhibitor peptide may comprise an antibody or fragment thereof, such as a single chain (scFv) fragment thereof, with specificity for said binding site of the protective antigen moiety.

scFv fragments with affinity for a selected antigen, such as said binding site, may be produced in accordance with methods known in the art, including phage or yeast display techniques. Such methods are described, for example, in Marks et al., 1991 Bypassing immunisation: human-antibodies from V-gene libraries displayed on phage *J.Mol.Biol.* 222: 581-597; and Vaughan TJ et al., 1996 Human antibodies with subnanomolar affinities isolated from a large non-immunised phage display library *Nat. Biotechnol.* 14: 309-314. According to such methods, a naïve antibody library may be synthesized from non-immunised human rearranged V-genes, and expressed in the form of fusion polypeptides with phage or yeast structural proteins for analysis of the affinity of members of the library for selected antigens. Typically, such methods enable the isolation and cloning of scFv fragments which bind to a selected antigen with a dissociation constant, K_d, in the nanomolar range. scFv fragments with improved antigen affinity may be

obtained through optimal kinetic screening of randomly mutagenised libraries of 10^5-10^7 yeast surface-displayed antibodies (Boder E et al., 2000 Direct evolution of antibody fragments with monovalent femtomolar antigen-binding affinity *PNAS* 97(20) 10701-10705). This method enables the cloning of single-chain antibody mutants having an antigen-binding equilibrium dissociation constant K_d in the femtomolar range, and displaying slow dissociation kinetics (dissociation half-time in the order of several days).

Alternatively, said PA binding inhibitor peptide may comprise a receptor mimetic peptide which is structurally homologous to a polypeptide which binds to said binding site in vivo, such as a receptor. Thus, where said PA binding inhibitor peptide is adapted to inhibit binding of said protective antigen moiety to a receptor on eukaryotic cells, said PA binding inhibitor peptide may comprise a peptide which is structurally homologous to the PA binding site of said receptor, such that said PA binding inhibitor peptide will bind said protective antigen moiety in competition with said receptor, thereby blocking binding of said protective antigen moiety to said receptor.

In another aspect of the invention, said PA binding inhibitor peptide may comprise a PA mimetic peptide which is structurally homologous to a binding site of said protective antigen moiety, such that said PA mimetic peptide is capable of competing with said protective antigen moiety for binding to any antigen or receptor which binds said binding site. Thus, for example, said PA binding inhibitor peptide may comprise recombinant PA or a fragment thereof.

In some embodiments of the invention, at least one of said one or more non-structural proteins adapted to inhibit anthrax toxin activity may comprise at least one LF/EF inhibitor peptide which is adapted to inhibit a cytotoxic catalytic activity, such as a proteolytic activity, of said anthrax

toxin. In particular, said LF/EF inhibitor peptide may be adapted to inhibit a cytotoxic catalytic activity of the lethal factor moiety or edema factor moiety of said anthrax toxin.

Thus, said LF/EF inhibitor peptide may be adapted to bind to an active site of said lethal factor moiety or edema factor moiety, such as to inhibit a cytotoxic catalytic activity mediated by said active site. Accordingly, said LF/EF inhibitor peptide may comprise an antibody or fragment thereof, such as a single chain (scFv) fragment thereof, with specificity for said active site of the lethal factor moiety or edema factor moiety.

Studies have shown that the toxicity of anthrax toxin may principally be attributed to the activity of lethal toxin; and accordingly, in preferred embodiments, said LF/EF inhibitor peptide may be adapted to inhibit a cytotoxic catalytic activity of the lethal factor moiety of said anthrax toxin. As reported in Klimpel KR, Arora N, and Leppla S, 1994 Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity *Mol Microbiol* 13: 1093-1100, lethal factor is a Zn-metalloprotease which has an active site located within domain 4 at the C-terminus of LF. The polypeptide sequence of the lethal factor moiety of *B. anthracis* is set out in Figure 1, where the peptide sequence His Glu Phe Gly His, which is known to be within the active toxicity site of lethal factor, is highlighted in bold. Advantageously, said LF/EF inhibitor peptide may be adapted to bind to said active toxicity site, such as to inhibit the cytotoxic activity mediated by said active site.

In one aspect of the invention, said recombinant bacteriophage may be adapted to express a first non-structural protein which is capable of causing lysis of anthrax-causing bacteria and a second non-structural protein, distinct from said first non-structural protein, which is adapted to inhibit anthrax toxin activity. Thus, the anti-anthrax activity of said recombinant

bacteriophage will be mediated by at least two independent protein molecules.

In another aspect of the invention, said recombinant bacteriophage may be adapted to express a non-structural protein comprising one or more polypeptides capable of lysing anthrax-causing bacteria fused with one or more polypeptides adapted to inhibit anthrax toxin activity. This will mean that both a bactericidal and a toxin-inhibitory function will be mediated by a single recombinant non-structural protein produced by said bacteriophage.

In some preferred embodiments of the invention, said bacteriophage is adapted to express at least one non-structural protein which comprises at least two polypeptides adapted to inhibit anthrax toxin activity, which at least two polypeptides include both a PA binding inhibitor peptide and an LF/EF inhibitor peptide. This will enable the bacteriophage to inhibit both the binding activity of the protective antigen moiety of said anthrax toxin, and the catalytic cytotoxic activity of the lethal factor or edema factor moiety of said anthrax toxin, hence ensuring efficient inhibition of said anthrax toxin activity.

In most especially preferred and particularly advantageous embodiments, said bacteriophage is adapted to express at least one non-structural protein which comprises a polypeptide capable of lysing anthrax bacteria, a PA binding inhibitor peptide, and an LF/EF inhibitor peptide. Accordingly, said bacteriophage may preferably be adapted to express a lysin with specificity for said anthrax bacteria, and scFv fragments which are respectively adapted to bind to a binding site on said protective antigen moiety and to an active site on said lethal factor moiety or said edema factor moiety. This will enable highly efficient anti-anthrax action.

In preferred embodiments, said bacteriophage may be modified so as to delete or inactivate one or more genes of said bacteriophage which are required for the lysogenic cycle of the bacteriophage. Thus, said

bacteriophage may be incapable of entering or sustaining the lysogenic cycle. Accordingly, said bacteriophage may be driven to enter the lytic cycle, thereby causing rapid death of infected anthrax bacteria.

Typically, said anthrax bacteria may comprise *Bacillus anthracis*, or any related anthrax bacterial strain, including variant strains which may arise by chance or deliberate mutation of known anthrax bacterial strains.

The administration of said phamaceutical composition to a patient in need of treatment for anthrax infection may be by way of oral, sublingual, transdermal, respiratory or parenteral administration.

Said effective amount of the pharmaceutical composition will depend on factors such as the nature and severity of the disorder being treated and on the weight, age and condition of the patient.

For oral or parenteral administration, it is greatly preferred that the pharmaceutical composition is administered in the form of a unit-dose composition, such as a unit dose oral or parenteral composition.

Such compositions are prepared by admixture and are suitably adapted for oral or parenteral administration, and as such may be in the form of tablets, capsules, oral preparations, powders, granules, lozenges, reconstitutable powders, injectable and liquid infusible solutions or suspensions or suppositories.

Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tabletting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well known methods in the art.

Suitable fillers for use include cellulose, mannitol, lactose, trehalose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycolate. Suitable lubricants include, for example, magnesium stearate. Suitable

pharmaceutically acceptable wetting agents include sodium lauryl sulphate.

These solid oral compositions may be prepared by conventional methods of blending, filling or tabletting. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents.

Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating.

For parenteral administration, fluid unit dose forms may be prepared comprising a sterile vehicle. The components of the composition, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the components of the composition in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. To enhance the stability, the composition may be frozen after filling into the vial and the water removed under vacuum.

Parenteral suspensions are prepared in substantially the same manner except that the compound may be suspended in the vehicle instead of being

dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent may be included in the composition to facilitate uniform distribution of the compound of the invention.

As is common practice, the compositions will usually be accompanied by written or printed directions for use in the treatment concerned.

Following are descriptions, by way of example only, and with reference to the accompanying Figures, of embodiments of the invention.

Figure 1 shows the polypeptide sequence of lethal factor of *Bacillus anthracis*, with the active site thereof highlighted in bold.

Figure 2 shows the polypeptide sequence of protective antigen of *Bacillus anthracis*, with the receptor-binding site highlighted in bold.

Description 1 : Cloning an scFv fragment capable of inhibiting metalloprotease activity of anthrax toxin lethal factor (LF/EF inhibitor peptide)

1.1 Isolation of anthrax toxin lethal factor

Bacillus anthracis Sterne strain (7702, Paseur Collection) was cultured in brain heart infusion medium (BHI; Difco, Detroit, Mich.) containing ampicillin (100μg/ml), spectinomycin (60 μg/ml), kanamycin (40 μg/ml) and erythriomycin (5 μg/ml). The culture was grown to OD_{600} = 0.7 and contained PA, LF and EF (Brosnier F, Weber-Levy M, Mock M, and Serard J-C, 2000 Role of Toxin Functional Domains in Anthrax Pathogenesis *Infec Immun* 68(4): 1781-1786). 5 l of culture is typically used for accumulation of cell suspension. Cells were removed by centrifugation at 4°C, protein precipitated with 75% ammonium sulphate and the precipitate was dissolved in 100 ml of 50mM phosphate buffer, pH 7.8 containing 300

mM NaCl. LF was absorbed onto Ni-nitriloacetic acid resin equilibrated and washed with the same buffer.

Protein was eluted with the gradient of imidasole chloride (0-500mM) and fractions containing LF were pooled, dialysed against TE buffer (10 mM Tris, 5mM EDTA, pH 8.0) overnight and concentrated on Centricion-30 (Amicon) to a volume 5 ml. The concentrate was loaded on a Sephacryl S-200 column equilibrated with the same buffer. Fractions containing LF were pooled and loaded on Mono-Q column (Pharmacia) equilibrated with TE buffer and protein was eluted with a linear gradient of NaCl (0-1M) in the same buffer. By this procedure LF can be purified to homogeneity according to SDS-PAGE with the gel stained with Coomassie blue (Gupta et al., 1998 : Expression and Purification of the Recombinant Lethal Factor of Bacillus Anthracis *Inf Immun* 66(2): 862-865).

Flow-through fractions obtained after chromatography on Ni-nitriloacetic acid resin were used for purification of PA antigen as indicated in Description 2.1 below.

1.2 Preparation of candidate clones

A library of single chain antibody fragments (scFvs) was first developed against the peptide domain of *B. anthracis* LF that is known to be a part of the LF catalytic domain (Klimpel KR, AroraN, and Leppla S, 1994 Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity *Mol Microbiol* 13: 1093-1100). The polypeptide sequence of lethal factor is set out in Figure 1, where the active site is indicated in bold.

A peptide fragment RNDSEGFI**HE FGHAVDDYAG** YLLDK was synthesized according to known methods for peptide synthesis utilising an Applied Biosystems 430A synthesizer following manufacturer's recommended procedures, and was used to generate an initial library of scFv

fragments, as described in Marks et al, 1991, *J.Mol.Biol.* 222:581-597 and Vaughan et al, 1996 *Nat. Biotechnol.* 14:309-314.

The library was screened as described in Description 1.3 below, so as to identify scFv fragments capable of inhibiting LF, and these fragments were used to generate a secondary library of scFv, having higher binding affinity to the LF catalytic site with dissociation constants in the picomolar range.

1.3 Analysis of LF-inhibitory function of candidate scFv clones

A fluorescence assay of the catalytic activity of LF –metalloprotease was designed, using a polypeptide having the peptide sequence RNDSEGFI**HEFGH**AVDDYAG (which is known to be cleaved by LF), The polypeptide was synthesised according to known methods of peptide synthesis, using an Applied Biosystems 430A synthesizer following manufacturer's recommended procedures. The polypeptide was then purified by reverse-phase HPLC; and its mass was verified by mass spectrometry.

Thereafter, the polypeptide was modified to encompass an N-terminal fluorescence donor and a corresponding C-terminal fluorescence acceptor. Suitable pairs of fluorescence donors/acceptors include 6-carboxyfluorescein/6-carboxytetramethylrhodamine and GFP (Green fluorescent protein) / BFP (Blue fluorescence protein) (Xu et al., 1998 Detection of programmed cell death using fluorescence energy transfer *Nucleic Acid Research* 26(8): 2034- 2035).

In the uncleaved fluorescence-modified polypeptide, the fluorescence spectrum of the N-terminal donor overlaps with the excitation spectrum of the C-terminal acceptor and the excitation of the donor induces the fluorescence of the acceptor, resulting in fluorescence resonance energy transfer (FRET). However, the FRET effect is very sensitive to the distance between the donor and acceptor and, on cleavage of the polypeptide, there is

a detectable decrease in fluorescence. This enables the ability of candidate scFvs to inhibit the cleaving activity of LF to be conveniently monitored.

Thus, the fluorescence-modified polypeptide was mixed with lethal factor prepared in accordance with Description 1.1 above, and with a candidate scFv, prepared in accordance with Description 1.2 above; and the effect on fluorescence relative to suitable controls was assessed. A decrease in the drop in fluorescence observed on addition of lethal factor to the polypeptide indicates effective inhibition of the cleavage activity of lethal factor by the candidate scFv.

scFvs capable of inhibiting lethal factor identified in this manner were accumulated in larger quantity, purified on DEAE-Sephadex and further investigated for their ability to inhibit LF activity in a mouse model of infection. The scFv clone with the highest activity in vivo and in vitro was then selected for fusion into phage DNA.

Measurements of fluorescence were made on a 96 well plate in a fluorescence spectrophotometer (model 850, Hitachi, Japan) with excitation at 492 nm. All measurements were made in a 10 mM Tris buffer.

Description 2 : Cloning an scFv fragment capable of inhibiting binding of anthrax toxin protective antigen to mammalian cell receptors (PA binding inhibitor peptide)

2.1 Isolation of anthrax toxin protective antigen

Native protective antigen was isolated from a culture of of PXO2-cured Sterne strain of *B.anthracis* by precipitation with 20% ammonium sulphate, resuspension of the precipitate and two step chromatography with the first step being ion-exchange using Mono-Q and the second step being gel-filtration using Sephadex G75 (Cirino NM et al., 1999 Disruption of Anthrax Toxin Binding with the Use of Human Antibodies and Competitive

inhibitors *Infection and Immunity* 67(6) 2957-2963). The fraction containing PA was then concentrated by the dead-end membrane filtration on Amicon Model 3 stirred cell containing PM10 ultrafiltration membrane (10,000 molecular weight cut-off). The purity of the final PA preparation was > 90% as determined by SDS-PAGE.

2.2 Preparation of candidate clones

A library of single chain antibody fragments (scFvs) was first developed against the peptide domain of *B. anthracis* PA that is known to be a part of the PA binding domain (Brosnier F, Weber-Levy M, Mock M, and Serard J-C, 2000 Role of Toxin Functional Domains in Anthrax Pathogenesis *Infec Immun* 68(4): 1781-1786). The polypeptide sequence of protective antigen is set out in Figure 2, where the binding site is indicated in bold.

A peptide fragment **PNYKVNVYAV TKENTIINPS ENGDTSTNGI KKILIFSKKG** was synthesised according to known methods for peptide synthesis utilising an Applied Biosystems 430A synthesizer following manufacturer's recommended procedures, and was used to generate an initial library of scFv fragments, as described in Marks et al, 1991, *J.Mol.Biol.* 222:581-597 and Vaughan et al, 1996 *Nat. Biotechnol.* 14:309-314.

The library was screened as described in Description 2.3 below, so as to identify scFv fragments capable of inhibiting PA binding, and these fragments were used to generate a secondary library of scFv, having an improved ability to inhibit PA binding.

2.3 Analysis of LF-inhibitory function of candidate scFv clones

Binding of PA to mammalian cell membranes can be detected by immunofluorescent microscopy of AlexoFluor-PA conjugate binding to a layer of THP-1 monocyte cells, and by flow cytometry assay using a

suspension of U937 monocyte/macrophage cells and AlexaFluor-PA conjugate.

Characterisation of PA binding to scFv fragments can be achieved using the BIAcore 2000 System (Pharmacia Biosensors) after purified PA was bound to BIAcore CM5 chip.

To study the inhibition of PA binding to cell receptors, various concentrations of scFv were added to each system under investigation to cover 1:1 and 10:1 molar ratios of PA and scFv.

Description 3 : Cloning a phage lysin capable of lysing anthrax bacteria

3.1 Isolation of phage

Isolation of new phage can be carried out in one of two ways – the activation of lysogenised phage in a collection of *B.anthracis* or *B.cereus* strains, or by collection of phage from the environment by established techniques, for example, as described in Smimizu et al (1998) *Japanese Journal of Food Microbiology* 15:147-152. A typical isolation protocol would be as follows (adapted from Parish and Stoker, Methods in Molecular Biology vol 101):

Gently resuspend solid sample (eg. soil) in 1 volume (w/v) phage buffer and store at 4°C overnight. Remove insoluble debris by centrifugation at 5000g for 10 min at 4°C. Carefully remove the supernatant and pass the sample through a 0.45îm filter, then through a 0.2îm filter saving aliquots of the original supernatant and each filtrate. Determine the titre of each sample.

For enrichment prior to titring, add 100g or 100ml of starting material into 1 litre of an early-log phase culture of *B.cereus* or *B.anthracis*. Grow culture normally until late-log or early stationary phase. Proceed as above from the centrifugation step.

Phage can be isolated using either *B.cereus* or *B.anthracis* as a target

strain. Phage should be tested on both strains before proceeding with further lysin characterisation to ensure that the lysin will be of interest.

3.2 Identification of lysin genes

Phage lysin genes are isolated using data from purified lysin N-terminal sequence data. The first step in this procedure is to purifiy phage lysin. Partial purification may be achieved using the following protocol:

Grow *B.cereus* to an OD_{600} of 0.3. Add bacteriophage to a ratio of cells:phage of between 1:2 and 1:10. Incubate the infection mix at 37°C for approximately 1-2 hours to enable phage lysis and liberation of lysin. Add DNAse to a final concentration of 50ìg/ml and incubate at 37°C for a further 30 min. Centrifuge the lysed culture at 5000g, 4°C, 20 min to remove cell debris. Lysins from other species vary in size in the range of 20-50 kDa. As a first step, removal of proteins above 50kDa using size exclusion or gel filtration. This step will eliminate phage from the preparation, an essential step to ensure that observed lytic activity is due to extracellular lysin rather than phage infection.

Partially purified lysin can at this stage be used for specificity testing. This procedure will involve growing *B.anthracis* test strains in liquid culture to an OD_{650} of approximately 0.8..400ìl of this culture is then mixed with sheep blood soft agar mix (0.7% agar) and poured onto sheep blood plates and left to set (10-15 min). 10ìl of purified lysin is then applied to the top of the soft agar layer and the plate incubated at 37°C until a homogenous layer of bacterial growth is in evidence. Active lysin is evident as a clear zone in the top agar layer, amongst confluent bacterial growth.

3.3 Cloning of candidate gene(s)

N-terminal sequencing is carried out on purified protein. Where purified protein from the previously described step does not yield protein of

sufficient purity for this procedure, further purification by SDS-PAGE is required. Lysin activity is associated with an individual band and is detected by overlay of a non-denaturing gel upon a soft-agar layer of *B.anthracis* (or *B.cereus*).

N-terminal sequence data from either the native protein or its cleavage product (in the case of N-terminal blockage or excessive redundancy in the back-translated sequence) facilitates cloning. The data from protein sequencing is used to isolate the encoding gene in one of two ways:

(i) Generate a degenerate oligonucleotide primer based on the N-terminal sequence and known *Bacillus* codon bias information and use in combination with a random 3' primer to directly PCR from the phage genome.

(ii) Use the degenerate probe to screen a restriction digest or library of the phage genome. Identified restriction fragments would be cloned and sequenced, identified library clones would be sequenced.

An alternative strategy for lysin gene cloning is outlined in Loessner et al (1997) J. Bacteriol. 179: 2845-2851. In this strategy, used to clone *B.cereus* phage lysins, the phage genome is partially digested and 1-3kb fragments cloned into an inducible *E.coli* expression vector. The resulting library is replica plated onto IPTG containing plates. After induction, the bacteria are lysed with chloroform and the presence of lysin detected by overlay with a concentrated layer of *B.cereus* in a soft agar lawn. Regions of clearing in the soft agar layer identify clones expressing lysin and these are isolated and the clone sequenced.

A large collection of bacillus bacteriophages as well as newly isolated environmental samples are to be used to generate expression "phage libraries" using the protocol describe above. The systematic screening and identification of Bacillus sp. phage lysins is to be undertaken

in order to identify candidates lytic enzymes to be used in the final recombinant phage (rphage).

The final rphage will express at least two distinct lytic enzymes in order to reduce any possibilities of emerging mutant host resistant to the phage lysin.

3.4 Overexpression in *E.coli*

Once sequenced, lysin gene(s) are amplified with appropriate primers to facilitate sub-cloning into suitable expression vectors (eg. Novagen pET or Invitrogen pAraBAD vector systems).

B121 (De3) *E.coli* cells transformed with the vector encoding lysin were grown overnight at 37°C in a defined medium to OD_{600} = 1.0. Cells were removed from the culture by centrifugation at 15,000xg for 20 min, and the lysin was concentrated 10-fold in a retentate with an Amicon Model 3 stirred cell containing PM10 ultrafiltration membrane (10,000 molecular weight cut-off). Typically 100 ml of concentrated retentate was used to recover phage lysin by precipitation with 90% ammonium sulphate and dissolving precipitate in 20 ml of Tris buffer (0.1M Tris, 0.1M NaCl, pH = 7.0). The concentrate was then loaded onto a gel-filtration column (Pharmacia) containing 250ml of Sephacryl S-200 equilibrated with the same buffer and eluate fractions were analysed for their lytic activity on the lawn of *M.smegmatis*. Active fractions were pooled, and loaded onto an ion-exchange column containing 10 ml of Mono-Q resin and protein fractions were eluted with the gradient of NaCl (0.1-0.5M) and analysed for their lytic activity on a lawn of *M.smegmatis*. The purity of lysin was assessed by PAGE-SDS electrophoresis using 12% gels and a Mini-Protean II electrophoresis system (BioRad). To identify the lysin band, the sample obtained after ion-exchange chromatography was divided into two and one part was incubated with a concentrated suspension of *M.smegmatis* at 4°C

for 1h to bind lysin and remove it from the sample. An absence of lysin activity was indicated by an absence of lytic zones on a *M.smegmatis* lawn loaded with few 10 il of the treated sample, in contrast with a lawn loaded with untreated sample. Then both samples were run by SDS-PAGE, bands were developed with Coomassie blue and the band missing within the protein profile of the treated sample and present in non-treated sample was identified. This band was extracted and its N-terminal sequence was analysed.

3.5 *In vitro* testing

Efficacy testing of bacterial lysis to determine required dose. Liquid culture experiments can be used to determine the efficacy of lysin induced killing of *B.anthracis* in terms of the amount of lysin required to eliminate a known quantity of bacteria. Tests are also carried out to determine the stability of purified lysin and to determine a suitable formulation strategy for inhalation.

Example 1. Development of recombinant phage capable of producing additional phage lysin and scFv peptides that can block PA binding and LF enzymatic activity

Phage, prepared as described in Description 3.1 above, is accumulated to 10^{12} pfu/ml and phage DNA is isolated by the standard method (Sambrook et al., Molecular cloning A Laboratory Manual, Second Edition). A phage label is randomly cloned into the phage genome, and a library is generated in which the label is inserted at every possible position throughout the phage genome.

The label encodes a detection epitope (S protein in the example given) which enables immunodetection of modified phage. The library is generated in a phasmid form of the phage genome so that it can be

propagated in *Escherichia coli* for amplification (prior to transformation of host) and for storage purposes.

At this stage the library contains all possible combinations of phage genome containing the insert. A selection procedure is required in order to identify viable modified phage and further, to identify phage displaying the required phenotype. The first step in this process is the transformation of *Bacillus cereus* with the phage library. This is achieved by electroporation and plaques only arise from viable recombinant phage (and non-modified wild type phage). The next step, to identify recombinant phage expressing the modifying epitope at the phage surface, is done by transferring to nitrocellulose membrane by the familiar plaque lift technique, and by screening the membrane for the presence of the S protein label by immunodetection (using HRP-linked anti-S protein antibodies).

The isolated phage are then recovered, propagated and analysed by sequencing, Southern blotting or restriction mapping, to identify the viable sites for surface modification. scFv encoding fragments and/or lysin-encoding genes, identified as described in Descriptions 1-3 above, are then inserted at the positions identified and viable phage are isolated in the same way, this time using an HRP-linked anti-scFv antibody.

The above described procedure is also used to determine sites in the phage genome that will accommodate larger fragments of DNA for insertion of an scFv expression cassette. The procedure is essentially the same but there is no need to screen for surface exposure. In brief, an *E.coli* plasmid will be introduced in place of the small label/internalisation element described above. A useful plasmid in this respect might be pBR322 or smaller derivatives thereof. Transformation of *E.coli* with the bacteriophage-plasmid ligation mix will select for genomes containing the *E.coli* plasmid. Back-transformation of DNA prepared from these candidates will select for phasmids containing the phage genome with the successfully integrated

plasmid. This procedure identifies sites in the phage genome which tolerate insertions of relatively large fragments of foreign DNA and further serve as the starting material for insertion of the scFv encoding DNA fragment. This is either cloned in place of or within the integrated plasmid along with an appropriately placed *Bacillus* promoter and ribosome binding site for proper expression of the scFv protein.

In both cases, expression of active scFv, either as a fusion or as an independently expressed protein, is confirmed by both *in vitro* testing (to ensure that the expressed scFv effectively binds PA or LF) and *in vivo* (to ensure that the scFv is efficacious in the disease situation).

In order to enhance the neutralizing capabilities of the recombinant scFv, whether in the form of a fusion or as an independently expressed protein , the open reading frame (ORF) will be placed under the regulation of strong phage promoter such as pLeft. The construct including the strong promoter upstream of the scFv can be cloned double or in multiple tandem in order to augment the production of the inhibitory peptide. The number of copies is to be determined by the stability of the rphage.

Phage label sequence

1 GAGCTCGGCG GCGGCGGCTC GATCGAGGGC CGCGGCGGCC

41 AGCCGGAGCT GGCCCCGGAG GACCCGGAGG ACATCGAGGG

81 GCGGGGCGGC AAGGAGACCG CCGCCGCCAA GTTCGAGCGC

121 CAGCACATGA ACTCGGCGGC CGCCGGCGCG CC

Translated below:

*Sac*I	Spacer	Xa	HSV	Xa
EL	GGGGS	IEGRGG	QPELAPEDPED	IEGRGG

S peptide	*Not*I	*Asc*I
GKRKKEMTKQKEVPKRVAKRKL		RRA

The tag detailed above is for illustrative purposes only, and is used to identify phage genome sites which enable in-frame insertion of peptides with little or no discernible disruption to phage function and viability. These sites are also selected to ensure surface exposure and full functioning of the inserted peptide. The phage label has complementary sites to facilitate the insertion of other fragments. Sites have been added in such a way as to keep the gene in-frame with both upstream and downstream fusions. For the *Not*I and *Asc*I sites this means adding an additional base. These additions have been made to minimise the charge differences and/or disruptions in flexibility or hydrophobicity (eg. AAA in *Not*I site –small aa). The label contains no stop codons to facilitate fusion at both 5' and 3' ends.

At least one of the essential genes required for the lysogenic cycle of the phage is to be either deleted or mutagenized in order to eliminate any subsequent possibilities of lysogeny. Lysogenic phages rely on a strong repressor system to suppress the lytic cycle of temperate bacteriophages. Production of mutant phages which are intrinsically lytic can be generated through serial passage *in vitro* using either *B. cereus* or the attenuated *B. anthracis* sterne strain as phage-host. Selection of lytic mutants displaying higher levels of activity are identified through plaque size quantification following an overnight incubation on a Bacillus lawn. Lytic activity of the selected mutant phages is to be quantified spectrophotometrically at an OD_{590} using host cell lysis as an indication of lytic actitvity. The mutations associated with the modified lytic phages are to be determined using

comparative genomic analysis of the wildtype phage and the mutant phage.

In parallel to the random mutagenesis process described above, deletions or modification of essential gene(s) required for lysogeny will be generated through homologous recombination of selected target sequence(s). Repressor gene(s) involved in the down regulation of lytic functions through the interaction with the promoters of the early lytic cycle are primary targets to be disrupted in order to prevent lysogeny. Theses genomic sequences are to be identified through i) comparative genomic analysis of the Bacillus phage and existing deposited DNA sequences available through the National Center Biotechnology Information (National Institute of Health, USA; http://www.ncbi.nlm.nih.gov/) , ii) comparative peptide/peptide structural analysis and predictions using the Swiss Proteomics database (Expert Protein Analysis System, Molecular Biology Server; http://www.expasy.ch/), iii) analysis of the genomic structure of the phage (for examples see Hendrix et al., 19999. Proc. Natl. Aca. Sci. 96: 2192-2197.), iv) through the identification of a protein-DNA complex involving the bacteriophage DNA and recombinant phage proteins generated through a phage- expression library. The DNA-Protein complex can be identified by gel-shift mobility or footprinting analysis as described by Sambrook and Russell , Molecular Cloning; Cold Spring Harbor Laboratory Press, 2001.

All PCR methods are carried out using Roche Diagnostics Hi-Fidelity™ DNA polymerase system using manufacturer's instructions. PCR products are cloned using the Invitrogen TOPO-TA™ cloning kit into vector pCR2.1 TOPO.

E.coli strain JM109 (New England Biolabs) or TOP10 (Invitrogen) are used to propagate plasmid constructs. Strain BL21(DE3) or HMS174(DE3) are used for *E.coli* expression using pET vectors.

Standard methods are used for all molecular biology and microbiology techniques, as described in Sambrook et al (Sambrook, Fritsch,

Maniatis "Molecular Cloning – A Laboratory Manual, 2nd Edition) and Parish and Stoker ("Methods in Molecular Biology 101: Mycobacteria Protocols").

CLAIMS

1 A recombinant lytic bacteriophage, said bacteriophage being adapted to express at least one non-structural protein which is adapted to cause lysis of anthrax bacteria and at least one non-structural protein which is adapted to inhibit anthrax toxin activity.

2 A bacteriophage as claimed in claim 1, which bacteriophage is specific for and lytic to said anthrax bacteria.

3 A bacteriophage as claimed in claim 2, which comprises lytic anthrax gamma-phage or bacteriophage CP-51.

4 A bacteriophage as claimed in any preceding claim, wherein at least one of said non-structural proteins capable of causing lysis of said anthrax bacteria comprises the binding and catalytic domains of a phage lysin with specificity for said anthrax bacteria.

5 A bacteriophage as claimed in any preceding claim, wherein at least one of said non-structural proteins capable of causing lysis of said anthrax bacteria comprises a phage lysin with specificity for said anthrax bacteria.

6 A bacteriophage as claimed in any preceding claim, wherein at least one of said non-structural proteins capable of causing lysis of said anthrax bacteria comprises an antibiotic, such as penicillin, ciprofloxacin or doxycycline.

7 A bacteriophage as claimed in any preceding claim, wherein at least one of said non-structural proteins adapted to inhibit anthrax toxin activity

comprises at least one PA binding inhibitor peptide which is adapted to inhibit a binding activity of the protective antigen moiety of said anthrax toxin.

8 A bacteriophage as claimed in claim 7, wherein at least one of said PA binding inhibitor peptides is adapted to inhibit binding of said protective antigen moiety to eukaryotic cells such as macrophages and/or monocytes, such as to prevent the importation into said eukaryotic cells of one or more moieties of said anthrax toxin having cytotoxic activity, such as the lethal factor moiety or edema factor moiety of said anthrax toxin.

9 A bacteriophage as claimed in claim 7 or claim 8, wherein at least one of said PA binding inhibitor peptides comprises a peptide with high binding affinity for a binding site on said protective antigen moiety, which peptide is arranged to bind to said binding site such as to prevent the binding of other polypeptides, such as receptors, to said binding site.

10 A bacteriophage as claimed in claim 9, wherein at least one of said PA binding inhibitor peptides comprises an antibody or fragment thereof, such as a single chain (scFv) fragment thereof, with specificity for said binding site of the protective antigen moiety.

11 A bacteriophage as claimed in claim 9 or claim 10, wherein at least one of said PA binding inhibitor peptides comprises a receptor mimetic peptide which is structurally homologous to a polypeptide which binds to said binding site in vivo, such as a receptor.

12 A bacteriophage as claimed in any of claims 7-11, wherein at least one of said PA binding inhibitor peptides comprises a PA mimetic peptide which is structurally homologous to a binding site of said protective antigen

moiety, such that said PA mimetic peptide is capable of competing with said protective antigen moiety for binding to any antigen or receptor which binds said binding site.

13 A bacteriophage as claimed in claim 12, wherein said PA mimetic peptide comprises recombinant PA or a fragment thereof.

14 A bacteriophage as claimed in any preceding claim, wherein at least one of said non-structural proteins adapted to inhibit anthrax toxin activity comprises at least one LF/EF inhibitor peptide which is adapted to inhibit a cytotoxic catalytic activity, such as a proteolytic activity, of said anthrax toxin.

15 A bacteriophage as claimed in claim 14, wherein at least one of said LF/EF inhibitor peptides is adapted to bind to an active site of said lethal factor moiety or edema factor moiety, such as to inhibit a cytotoxic catalytic activity mediated by said active site.

16 A bacteriophage as claimed in claim 15, wherein at least one of said LF/EF inhibitor peptides comprises an antibody or fragment thereof, such as a single chain (scFv) fragment thereof, with specificity for said active site of the lethal factor moiety or edema factor moiety.

17 A bacteriophage as claimed in any preceding claim, which is adapted to express a first non-structural protein which is adapted for causing lysis of anthrax-causing bacteria and a second non-structural protein, distinct from said first non-structural protein, which is adapted to inhibit anthrax toxin activity.

18 A bacteriophage as claimed in any of claims 1-16, which is adapted

to express a non-structural protein comprising one or more polypeptides capable of lysing anthrax-causing bacteria fused with one or more polypeptides adapted to inhibit anthrax toxin activity.

19 A bacteriophage as claimed in any of claims 1-16, which is adapted to express one or more non-structural proteins which comprise a polypeptide capable of lysing anthrax bacteria, a PA binding inhibitor peptide which is adapted to inhibit a binding activity of the protective antigen moiety of said anthrax toxin, and an LF/EF inhibitor peptide which is adapted to inhibit a cytotoxic catalytic activity, such as a proteolytic activity, of said anthrax toxin.

20 A bacteriophage as claimed in any preceding claim, wherein said anthrax bacteria comprises *Bacillus anthracis*, or a related anthrax bacterial strain, such as a variant *Bacillus anthracis* strain which has arisen by chance or by deliberate mutation of *Bacillus anthracis*.

21 A pharmaceutical composition comprising a recombinant bacteriophage in accordance with any preceding claim and one or more pharmaceutically acceptable excipients or carriers.

22 A method for the treatment of anthrax in a host, comprising the step of administering to said host a recombinant bacteriophage in accordance with any of claims 1-20, which bacteriophage is lytic to at least one strain or to a mixture of strains of bacteria within said host.

23 Use of a bacteriophage in accordance with any of claims 1-20 in the production of a medicament for use in the treatment of anthrax infection.

Figure 1

```
      1  MNIKKEFIKV ISMSCLVTAI TLSGPVFIPL VQGAGGHGDV
GMHVKEKEKN
     51  KDENKRKDEE RNKTQEEHLK EIMKHIVKIE VKGEEAVKKE AAEKLLEKVP
    101  SDVLEMYKAI GGKIYIVDGD ITKHISLEAL SEDKKKIKDI YGKDALLHEH
    151  YVYAKEGYEP VLVIQSSEDY VENTEKALNV YYEIGKILSR DILSKINQPY
    201  QKFLDVLNTI KNASDSDGQD LLFTNQLKEH PTDFSVEFLE QNSNEVQEVF
    251  AKAFAYYIEP QHRDVLQLYA PEAFNYMDKF NEQEINLSLE ELKDQRMLSR
    301  YEKWEKIKQH YQHWSDSLSE EGRGLLKKLQ IPIEPKKDDI IHSLSQEEKE
    351  LLKRIQIDSS DFLSTEEKEF LKKLQIDIRD SLSEEEKELL NRIQVDSSNP
    401  LSEKEKEFLK KLKLDIQPYD INQRLQDTGG LIDSPSINLD VRKQYKRDIQ
    451  NIDALLHQSI GSTLYNKIYL YENMNINNLT ATLGADLVDS TDNTKINRGI
    501  FNEFKKNFKY SISSNYMIVD INERPALDNE RLKWRIQLSP DTRAGYLENG
    551  KLILQRNIGL EIKDVQIIKQ SEKEYIRIDA KVVPKSKIDT KIQEAQLNIN
    601  QEWNKALGLP KYTKLITFNV HNRYASNIVE SAYLILNEWK NNIQSDLIKK
    651  VTNYLVDGNG RFVFTDITLP NIAEQYTHQD EIYEQVHSKG LYVPESRSIL
    701  LHGPSKGVEL RNDSEGFI[HE] [FGH]AVDDYAG YLLDKNQSDL VTNSKKFIDI
    751  FKEEGSNLTS YGRTNEAEFF AEAFRLMHST DHAERLKVQK NAPKTFQFIN
    801  DQIKFIINS
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Figure 2

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   1  MKKRKVLIPL MALSTILVSS TGNLEVIQAE VKQENRLLNE SESSSQGLLG
  51  YYFSDLNFQA PMVVTSSTTG DLSIPSSELE NIPSENQYFQ SAIWSGFIKV
 101  KKSDEYTFAT SADNHVTMWV DDQEVINKAS NSNKIRLEKG RLYQIKIQYQ
 151  RENPTEKGLD FKLYWTDSQN KKEVISSDNL QLPELKQKSS NSRKKRSTSA
 201  GPTVPDRDND GIPDSLEVEG YTVDVKNKRT FLSPWISNIH EKKGLTKYKS
 251  SPEKWSTASD PYSDFEKVTG RIDKNVSPEA RHPLVAAYPI VHVDMENIIL
 301  SKNEDQSTQN TDSQTRTISK NTSTSRTHTS EVHGNAEVHA SFFDIGGSVS
 351  AGFSNSNSST VAIDHSLSLA GERTWAETMG LNTADTARLN ANIRYVNTGT
 401  APIYNVLPTT SLVLGKNQTL ATIKAKENQL SQILAPNNYY PSKNLAPIAL
 451  NAQDDFSSTP ITMNYNQFLE LEKTKQLRLD TDQVYGNIAT YNFENGRVRV
 501  DTGSNWSEVL PQIQETTARI IFNGKDLNLV ERRIAAVNPS DPLETTKPDM
 551  TLKEALKIAF GFNESNGNLQ YQGKDITEFD FNFDQQTSQN IKNQLAELNV
 601  TNIYTVLDKI KLNAKMNILI RDKRFHYDRN NIAVGADESV VKEAHREVIN
 651  SSTEGLLLNI DKDIRKILSG YIVEIEDTEG LKEVINDRYD MLNISSLRQD
 701  GKTFIDFKKY NDKLPLYISN PNYKVNVYAV TKENTIINPS ENGDTSTNGI
 751  KKILIFSKKG YEIG
```

INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/GB 02/03384

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N15/10 C12N7/01 C07K14/32 C07K16/12
A61K35/76 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, SEQUENCE SEARCH, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	M. MOUREZ ET AL.: "Designing a polyvalent inhibitor of anthrax toxin" NATURE BIOTECHNOLOGY, vol. 19, no. 10, October 2001 (2001-10), pages 958-961, XP002192493 NATURE PUBL. CO.,NEW YORK, US the whole document ---	
T	A.G. BECK-SICKINGER: "Anthrax: Terror im Immunsystem" NACHRICHTEN AUS CHEMIE UND TECHNIK, no. 1, January 2002 (2002-01), pages 42-45, XP002192494 Zeitschrift der Gesellschaft Deutscher Chemiker, Weinheim, DE the whole document --- -/—	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 2002	11/11/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BAILLIE L W J ET AL: "A heat-inducible Bacillus subtilis bacteriophage PHI105 expression system for the production of the protective antigen of Bacillus anthracis." FEMS MICROBIOLOGY LETTERS, vol. 163, no. 1, 1 June 1998 (1998-06-01), pages 43-47, XP002218776 ISSN: 0378-1097 the whole document	
A	WO 98 05344 A (BRIGHAM & WOMENS HOSPITAL ;SARKAR SAUMYENDRA N (US); DUBIN DANIEL) 12 February 1998 (1998-02-12) the whole document	
A	WO 00 61190 A (MICROBIOLOGICAL RES AUTHORITY ;PASECHNIK VLADIMIR ARTYMOVICH (GB);) 19 October 2000 (2000-10-19) the whole document	
A	WO 01 50866 A (ALAVIDZE ZEMPHIRA ;PASTERNACK GARY R (US); BROWN TORREY C (US); IN) 19 July 2001 (2001-07-19) the whole document	
A	WO 00 02522 A (LEE JOHN S ;PUSHKO PETER (US); WELKOS SUSAN L (US); PARKER MICHAEL) 20 January 2000 (2000-01-20) cited in the application the whole document	
A	CIRINO NICK M ET AL: "Disruption of anthrax toxin binding with the use of human antibodies and competitive inhibitors." INFECTION AND IMMUNITY, vol. 67, no. 6, June 1999 (1999-06), pages 2957-2963, XP002185394 ISSN: 0019-9567 cited in the application the whole document	
A	WO 94 18332 A (US HEALTH) 18 August 1994 (1994-08-18) cited in the application the whole document	

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LOESSNER MARTIN J ET AL: "Three Bacillus cereus bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different Bacilli." JOURNAL OF BACTERIOLOGY, vol. 179, no. 9, 1997, pages 2845-2851, XP002185395 ISSN: 0021-9193 cited in the application the whole document	
A	KLIMPEL KURT R ET AL: "Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity." MOLECULAR MICROBIOLOGY, vol. 13, no. 6, 1994, pages 1093-1100, XP001041909 ISSN: 0950-382X cited in the application the whole document	
A	BROSSIER FABIEN ET AL: "Role of toxin functional domains in anthrax pathogenesis." INFECTION AND IMMUNITY, vol. 68, no. 4, April 2000 (2000-04), pages 1781-1786, XP002185396 ISSN: 0019-9567 cited in the application the whole document	
A	GUPTA PANKAJ ET AL: "Expression and purification of the recombinant lethal factor of Bacillus anthracis." INFECTION AND IMMUNITY, vol. 66, no. 2, February 1998 (1998-02), pages 862-865, XP002185397 ISSN: 0019-9567 cited in the application the whole document	
A	WO 00 50069 A (FISCHETTI VINCENT ;LOOMIS LAWRENCE (US); NEW HORIZONS DIAGNOSTICS) 31 August 2000 (2000-08-31) the whole document	

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BODER ERIC T ET AL: "Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 97, no. 20, 26 September 2000 (2000-09-26), pages 10701-10705, XP002185398 September 26, 2000 ISSN: 0027-8424 cited in the application the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/03384

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 22 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

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